Fluorogenic and Chromogenic Substrates Used in Bacterial Diagnostics

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INTRODUCTION

New techniques have been developed recently for detection and differentiation of bacteria. They are based on the utilization of chromogenic and fluorogenic substrates for detection of activities of specific enzymes. These sensitive methods have led to improved accuracy and faster detection and may be performed by using the primary isolation media, thus bypassing the need for time-consuming isolation procedures prior to identification.

Furthermore, judicious choice of the synthetic substrates permits several reactions to be examined simultaneously. This subject has been reviewed previously (15, 16, 237). This review deals primarily with recent publications, with partic-

ular emphasis on methods for specific detection of Escherichia coli.

DETECTION OF ENZYMATIC ACTIVITIES

General Considerations

Three groups of fluorogenic and/or chromogenic reactions have been used.

(i) The first is hydrolysis of synthetic substrates by bacterial enzymes, causing considerable increase in the fluorescence and/or absorption of the bacterium-substrate mixture (16). Fluorogenic synthetic enzyme substrates containing coumarin derivatives of 4-methylumbelliferone (4-MU) or 7-amino-4-methylcoumarin (7-AMC) are the most commonly used substrates. This popularity could be ascribed to availability of a wide range of substrates with different metabolic moieties, noncarcinogenicity, ease of visual detection of the

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products of enzyme activity with UV light sources, and availability of suitable fluorometers for measurement of fluorescence in both tube and multiwell panels. Dyer (54) was the first to suggest the use of fluorogenic substrates for the detection of bacterial enzymes. Dahlén and Linde (41) described an agar plate, and Maddocks and Greenan (142) described a 10-min spot test in which 4-MU derivatives were used. Fujiwara and Tsuru (71) were the first to use 7-AMC derivatives for measurement of bacterial peptidases. Bascomb (16) described in detail the synthetic moieties of both fluorogenic and chromogenic enzyme substrates and the application of these substrates to study of bacterial enzymes. The derivatives of the following substrates have since been used most frequently: esters of o-nitrophenol (ONP) (135) or p-nitrophenol (PNP) (31) (release of the nitrophenol results in an increase in A_{405} and appearance of a yellow color); esters of indoxyl (31) or 5-bromo-4-chloro-3-indolyl (244) (the released indoxyl causes a blue color in colonies or suspensions of organisms positive for hydrolyzing such esters [31, 137, 244]); esters of 4-MU (151) (the released 4-MU shows an increase in fluorescence [excitation wavelength, 365 nm; emission wavelength, 440 nm] [79], and blue fluorescence can be observed visually when plates or tubes are irradiated with long-wavelength UV light, i.e., a Woods lamp [142]) (The Baxter Diagnostics Inc. MicroScan [18] Rapid Gram-Negative and Gram-Positive Identification Panels described in 1987 [118] utilize 4-MU derivatives of monoand disaccharides and phosphates); aryl peptide derivatives of p-nitroaniline (p-NA) (32) (release of p-NA results in an increase in A_{405} and appearance of a yellow color [20, 35, 38, 61, 71]; a substantial increase in sensitivity can be achieved by reacting the p-NA with a diazo dye [16], yielding blue to dark purple colors [22, 109]); and arvl peptide derivatives of 7-AMC (71, 79) (release of 7-AMC results in an increase in fluorescence that is detectable spectrophotometrically [excitation wavelength, 370 nm; emission wavelength, 440 nm] [71] or by visual observation of increased blue fluorescence under UV light [81]).

(ii) The second group of reactions is the change in fluorescence or absorbance of a pH indicator in a mixture of bacteria and substrate, which is caused by specific enzymatic activity, e.g., an increase in pH resulting from urease activity (79). A number of fluorescent pH indicators with different pH optima are available (50, 82, 224). Dyer (54) suggested the use of 4-MU as an indicator for the detection of an increase in pH and quinine for the detection of a decrease. Godsey et al. (79) used 4-MU for the detection of urease activity. Tsien (224) describes the use of fluorescent pH indicators for intracellular studies of eucaryotic cells. The MicroScan Rapid Identification system (118) uses fluorescent pH indicators for the detection of urease activity, decarboxylation of lysine and ornithine, and acid formation from some mono- or disaccharides. Similar tests are also available in the Sensititre Identification system (Radiometer America Inc.).

(iii) The third group of reactions is the change in intensity of fluorescence as a result of adsorbance of fluorescent dye onto some component of the bacterial cell, e.g., acridine orange (AO) binding to DNA (174) and 8-anilino-1-naphthalene sulfonic acid (ANS) binding to proteins (185). Important extensions of this approach are the use of epifluorescence microscopy (133), direct epifluorescent filter technique (DEFT) (173), and flow cytometry (FCM) (152) for counting and characterization of cell populations. These techniques can be applied directly to microbial cells present in the specimen without preincubation or cell multiplication. Fix-

ation of the bacterial cells is frequently required to facilitate penetration of the dye. In these techniques, cell populations are stained with nucleic acid or other specific reagents. In DEFT, bacterial cells are preferentially concentrated by filtration. In FCM, cells are counted on the basis of particle size and volume and the fluorescence at specific excitation and emission wavelengths.

DETECTION OF ACTIVITY OF INDIVIDUAL ENZYMES

Glycosidases

β-D-Glucuronidase (GUD) (EC β-D-Glucuronidase. 3.2.1.31) is an enzyme that catalyzes the hydrolysis of β-D-glucopyranosiduronic (GLR) derivatives into their corresponding aglycons and D-glucuronic acid. The enzyme is believed to be involved in decomposition of the intercellular substances of the host's connective tissue (41). The prevalence of this enzyme and its utility in detection of E. coli were reviewed previously (16, 92). GUD-positive reactions are observed in 94 to 96% of E. coli isolates tested (41, 58, 66, 74, 91, 115, 120, 148, 228, 239). In contrast, GUD activity is less common in Shigella (44 to 58%), Salmonella (20 to 29%), and Yersinia strains and in flavobacteria (79, 91, 134, 169, 177, 191, 223). The test may be useful in identification of certain Salmonella species (134).

Among the gram-positive group, the test was shown to be useful in characterization of group B and D streptococci (193). Only some *Staphylococcus* (154) and *Clostridium* (201) strains exhibited positive GUD reactions.

Dahlén and Linde (41) used a plate method to demonstrate GUD activity in *Bacteroides* and *Corynebacterium* spp. They also showed that the major activities of a single *E. coli* strain and an anaerobic *Corynebacterium* strain were recovered in the intracellular fraction.

GUD activity can be measured by using different chromogenic and fluorogenic substances. Release of phenolphthalein from a phenolphthalein-mono-β-D-glucuronide complex (PHEGLR) (30), of PNP from p-nitrophenol-β-Dglucuronide (PNPGLR) (1, 91, 120, 121, 122, 123, 238), and of indigo from 5-bromo-4-chloro-3-indolyl-B-D-glucuronide (X-GLR) (49, 68, 111, 137, 236) has been used for the specific detection of E. coli. The most commonly used substrate is 4-methylumbelliferyl-β-D-glucuronide (MUGLR) (6, 9, 30, 41, 46, 58, 78, 89, 93, 99, 103, 106, 122, 143, 149, 150, 155, 159, 166, 172, 179, 192, 204, 213). This compound is hydrolyzed by GUD, yielding 4-MU, which shows blue fluorescence when irradiated with long-wave UV light (excitation wavelength, 365 nm). Primary isolation media may be used for indication of the presence of E. coli or for isolation of E. coli colonies. In the first instance, a change in absorption or fluorescence of the entire medium is a sufficient indicator. In the second case, it is important to maintain localization of the reaction product in or around the bacterial colony. Substrates which give insoluble colored or fluorescent hydrolysis products are preferable to those giving soluble fluorophores or chromophores.

Chromogenic and fluorogenic substrates have different advantages in testing procedures for *E. coli*. PNPGLR is suitable for liquid media used for estimation of numbers of *E. coli*, e.g., when applying a most-probable-number technique (123), but less suitable for solid media because of the extensive diffusion of the yellow color (49, 121, 137, 143). However, Henrichsen (94) showed that PNPGLR, incorporated in agar medium, could be used for presumptive identification of *E. coli* from urine samples. When X-GLR is

TABLE 1. Concentrations of MUGLR proposed for incorporation into primary isolation media

Medium	MUGLR concn (μg/ml)	Reference
Brilliant green bile		
Lactose broth	50	78
E. coli broth	50	191
Lauryl sulfate broth	50	148
•	100	66
MacConkey agar	150	223
Violet red bile agar	200	66

used, colonies with enzymatic activity show a purple color without diffusion (49, 137, 236); it is therefore most suitable for incorporation in solid medium.

MUGLR can be incorporated into either liquid or solid media. The minimum concentration sufficient for distinct indication was 50 μ g/ml (Table 1). However, the concentration necessary for acceptable detection of *E. coli* depends on the other constituents of the media. Because the fluorescence of 4-MU is pH dependent (82), the pH of growth media containing MUGLR should be neutral or slightly alkaline (69, 82); otherwise, alkaline solution must be added to reveal the fluorescence (69, 142).

Besides its specificity and applicability, MUGLR has further advantages. It can be sterilized together with other medium ingredients without loss of activity at concentrations of 50 (189), 100 (66), and 200 (154) µg/ml; furthermore, no inhibitory effect on E. coli growth has hitherto been observed (154). Several MUGLR testing procedures were proposed for identification and enumeration of E. coli. These include most-probable-number (9, 66, 123, 124, 143, 206), membrane filtration (64, 143), presence-absence test (136, 143), agar plate (41, 44, 143, 150), microtitration plate (66), paper strip (72, 90, 96), or cupule on a plastic strip (72) techniques. Some *Pseudomonas* strains produce a pigment that exhibits dull greenish fluorescence under long-wave UV light. This may mimic the excitation and emission spectra of 4-MU during measurement of fluorescence, but the fluorescence signal is usually too small for visual detection. Visual detection of the fluorescence reaction in the MUGLR media is improved by using either a dark room or a dark box such as a CAMAG UV cabinet to exclude most of the incident light. Several authors have reported the application of specific GUD media for the identification of E. coli from different sources (Table 2).

For selective detection of *E. coli* in primary isolation media, aerobic incubation and incorporation of substances

TABLE 2. Application of GUD media for identification and enumeration of *E. coli*

Synthetic moiety of substrate	References for sample source:		
	Clinical or veterinary specimen	Water or food	
Indoxyl ^a p-Nitrophenol 4-MU	49 91, 120, 121 36, 51, 79, 93, 108, 166, 220, 223	68, 186, 236 122, 123, 148 4, 6, 9, 11, 30, 66, 78, 89, 90, 103, 106, 122, 149, 150 154, 176, 188, 239	

^a Refers to indoxyl or to 5-bromo-4-chloro-3-indole.

which inhibit the growth of gram-positive strains are used to suppress GUD activities of other bacteria. Additionally, incubation at 44°C and detection of gas formation help in exclusive detection of E. coli. This test is used increasingly for detection of E. coli in specimens from environmental and food sources, as E. coli is an important indicator of fecal contamination in samples from food-processing and water purification plants. GUD activity is found in the majority of E. coli isolates. However, Chang et al. (36) studied the proportion of GUD-negative E. coli isolates in human fecal samples and found that an average of 15% of E. coli isolates were GUD negative in lauryl sulfate tryptose broth with MUGLR. Some E. coli strains were temperature dependent for GUD production (very weakly positive at 37°C, but strongly positive at 44°C). Thompson et al. (220), investigating the MUGLR activity of 1,483 isolates, showed very good correlation between verocytotoxin production and a negative MUGLR response. A 20-min test was developed that allows reliable prediction of verocytotoxin production by strains belonging to E. coli serotype O157:H7. The use of growth-dependent tests (including GUD) to identify cysteine-requiring strains of E. coli and other members of the family Enterobacteriaceae may lead to errors due to falsenegative results. This source of error can be remedied by using a cysteine-supplemented inoculum (140).

β-D-Galactosidase. β-D-Galactosidase (β-GAL) (EC 3.2.1.23), trivially called lactase, catalyzes the breakdown of lactose into galactose and glucose and has therefore been used mostly for enumerating the coliform group within the family *Enterobacteriaceae*. The activity of β-GAL was determined by using the following chromogenic substrates: *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (55–57, 134, 135, 219, 234), *p*-nitrophenyl-β-D-galactopyranoside (PNPGAL) (31), or 6-bromo-2-naphthyl-β-D-galactopyranoside (BNGAL) (31), and the fluorogenic substrate 4-methylumbelliferyl-β-D-galactopyranoside (MUGAL) (23, 33, 79, 85, 142).

The tendency of chromogenic nitrophenolic substances to diffuse through solid media was observed with both ONPG and PNPGAL. Therefore, agars containing these substrates cannot be used (137, 143). To circumvent this problem, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) is preferred for the rapid detection of coliforms (143, 184). Using MUGAL and following the membrane filtration technique, Berg and Fiksdal (23) detected as few as 1 CFU of fecal coliforms per 100 ml of water in 6 h. Incorporation of MUGAL into E. coli agar and lauryl sulfate broth enables the direct counting of coliforms by applying a membrane filtration or presence-absence test (145). The test for the presence of this enzyme was found useful in differentiating among aerobic actinomycetes (67). An indirect method for estimating fecal coliform concentration, by using ONPG hydrolysis, was described by Warren et al. (234). A linear correlation was shown between the most probable number and the time required to obtain half-maximum A_{420} by bacteria concentrated from water specimens by filtration. Koumura et al. (127) described a similar approach for the detection of small numbers of microorganisms in a variety of specimens by using 4-MU derivatives of β -GAL and phosphate.

6-Phospho-β-D-galactoside 6-phosphogalactohydrolase. 6-Phospho-β-D-galactoside 6-phosphogalactohydrolase (β-PGAL) (EC 3.2.1.85) and β-GAL in a number of lactic streptococci were examined by using the chromogenic substrates ONP-β-D-galactopyranoside-6-phosphate and ONP-β-D-galactopyranoside (156). The enzyme β-GAL could be detected in only one organism, Streptococcus lactis 7962;

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other lactic streptococci examined showed different amounts of β-PGAL activity but no β-GAL activity.

Vogensen et al. (230) used X-GAL for a direct differentiation on agar between *Leuconostoc cremoris* and *Lactococcus lactis*. Under these conditions, *L. cremoris* is able to split X-Gal, appearing as blue colonies, but *L. lactis* subsp. *diacetylactis*, *L. lactis*, and *L. lactis* subsp. *cremoris* do not hydrolyze X-Gal.

Simultaneous detection of GUD and β -GAL. In the quality control of water and food, it is highly important to examine both coliforms and $E.\ coli$. Viable counts of these bacteria are widely used as indicators of bacteriological quality as well as for detection of fecal pollution. Novel methods have been introduced that are based on the detection of specific enzyme activity (GUD and β -GAL) by using fluorogenic and/or chromogenic substrates. Thompson and Borczyk (219) described a single-tube medium enabling the detection of motility, of activities of β -GAL and phenylalanine-desaminase, and of production of indole, which is useful for screening for members of the family Enterobacteriaceae in enteric or other clinical specimens.

A considerable number of studies have used a combination of nitrophenol derivatives, for detection of β -GUD, and 4-MU or indoxyl derivatives, for detection of GLR activities; B-GAL is indicative of total coliforms, and GUD is indicative of E. coli, indicating fecal contamination (39, 55-57, 69, 136, 189). Such media are used in combination with most-probable-number, presence-absence, and other detection methods. Because a small percentage of E. coli strains may not show GUD activity, and because some food samples, e.g., oysters, may show endogenous GUD activity, other methods for specific detection of E. coli were sought. These include serological detection of β -GAL, GUD, and glutamate decarboxylase enzymes in cell lysates by using antibodies specific to these enzymes isolated from E. coli. The presence of these enzymes in the cell lysates can be detected by coagulation tests with polyclonal antibodies to the E. coli enzymes. Alternatively, antibodies specific to the E. coli B-GAL and GUD enzymes were attached to wells of microtitration plates and used to capture the cell lysate enzymes, whose activity was detected by using 4-MU or ONP derivatives (115).

α-p-Galactosidase. α-p-Galactosidase (α-p-GAL) (EC 3.2.1.22) is useful for differentiation of streptococci and enterococci (138). 4-MU-α-p-galactoside is hydrolyzed by Streptococcus bovis and Enterococcus faecium and its biotypes. A streptococcal selective medium containing a fluorogenic α-p-GAL substrate and colorimetric starch test was described previously (138). Three phenotypic groups are identifiable on the basis of starch hydrolysis and fluorescence: (i) S. bovis positive for both tests; (ii) E. faecium positive only for α-p-GAL; (iii) Enterococcus faecalis, Enterococcus avium, Streptococcus equinus, Streptococcus mitis, and Streptococcus salivarius negative for both tests.

β-D-Glucosidase. The use of β-D-glucosidase (β-D-GLU) (EC 3.2.1.21) for bacterial differentiation has been summarized previously (16). Its usefulness in differentiation of streptococci has been recently demonstrated (116, 165). 4-MU-β-D-glucoside in conjunction with PNP-α-D-galactopyranoside and in the presence of 2.5% sodium deoxycholate were used to identify S. bovis and related bacteria. This combination test, in conjunction with a rapid assay for L-pyrrolidonyl-aminopeptidase (see below), could distinguish S. bovis, S. equinus, Enterococcus spp., Streptococcus pneumoniae and the viridans group streptococci in 30 min.

A fluorogenic assay for the detection of β -D-GLU was developed as part of a simplified conventional method to distinguish *Staphylococcus warneri* and *Staphylococcus hominis* isolated from bovine body sites. The assay is based on the fact that strains of *S. warneri* produce β -D-GLU, while strains of *S. hominis* do not (242).

 α -Amylase and α -glucosidase. Starch hydrolysis and production of acid from starch have long been used for differentiation of enterobacteria and streptococci. However, only a few synthetic substrates are available for measurement of the activity of α -amylase. A mixture of PNP derivatives of penta-, hexa- or hepta-maltose was used by Trepeta and Edberg (222) for detection of bacterial α -amylase (EC 3.2.1.1). A new chromogenic substrate for the assay and detection of α -amylase was reported by Biely et al. (24). The substrate was prepared by coupling partially hydrolyzed starch with a dye (ostazin brilliant red H-3B). It is precipitable from buffered solutions with ethanol and is equally suitable for the assay of α -amylase isoenzymes in gels.

Assays for α -glucosidase (α -GLU) (EC 3.2.1.20) and α -maltosidase, in the presence of Triton X-100, can be used to distinguish *Bacillus anthracis* from *Bacillus cereus*, *Bacillus mycoides*, and *Bacillus thuringiensis* (199).

Neuraminidase. Acetylated amino sugars are important constituents of naturally occurring polysaccharides and glycoproteins. Sialidases (acetylneuraminyl-hydrolases) cleave an α -ketosidic linkage between sialic acid and the glycosyl residues of glycoproteins, glycolipids, or colominic acids.

A number of substrates are now available for detection of glycosidases specific to acetylamino sugars. They include ONP and 4-MU derivatives of β-D-galactosamine (β-GalNAc), β-D-glucosamine (β-GluNAc), 2-D-N-acetylneuraminic acid (α -NeuNAc), and β -D-N', N'-diacetylchitobiose. Activities of enzymes hydrolyzing these substrates were sometimes considered to be related to pathogenicity, e.g., neuraminidase (187), breakdown of chitinaceous material (161). ONP-β-GluNAc was used for differentiating between B. mycoides and B. anthracis (199). Sialidase (EC 3.2.1.18) activities were determined by using 4-MU-α-NeuNAc and a fluorogenic assay (183); a qualitative filter paper assay was applied to Actinomyces strains, with 79% of the strains showing activity (157). All 16 strains of Actinomyces viscosus showed activity. The tests are most useful for characterization of Vibrio, Serratia, and Flavobacterium species. Two of these tests are included in MicroScan Rapid Identification panels (18).

Kahane et al. (113) described the activities of α - and β -glucosidases, α -GAL, β -GAL, α - and β -mannosidases, fucosidase, N-acetylneuraminidase, and β -N-acetylglucosaminidase of mycoplasmas at acidic pH by fluorometry with the appropriate 4-MU substrate. By this method, it seems that each species has a unique enzyme profile of its own. However, they concluded that the enzyme tests used did not provide differentiation between pathogenic and nonpathogenic mycoplasmas. Whiley et al. (241) used 4-MU derivatives of a number of sugars and acetylamino sugars for characterization and identification of Streptococcus milleri group isolates.

Esterases and Lipases

Esterases are found in all living organisms, and the use of the diacetate ester of fluorescein for the detection of viability has been summarized by Bascomb (16). Tsien (224) discussed the factors determining the intracellular fluorescence of fluorescein. Esterases hydrolyze molecules with shorter-

chain organic acids, whereas lipases are capable of acting on derivatives of longer-chain acids. For detecting these enzymes, Guilbault and Kramer (87) described fluorescence tests in which they used fluorescein derivatives. Use of butyryl, hexanoyl, heptanoyl, nonanoyl, palmitoyl, and oleyl esters of 4-MU has also been described (53, 110, 198, 215). Guilbault et al. (88) compared the suitability of 12 different compounds as substrates for detecting various lipases. Pancholy and Lynd (164) described a method for detection of esterase-synthesizing microbial colonies within mixed-culture plates by using induced hydrolysis of butyryl-4-MU. A plate assay was further designed to detect bacterial lipases in a medium containing trioleylglycerol and the fluorescent dye rhodamine B. Substrate hydrolysis causes the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation (126).

Butyrate esterase has been found in cultures of *Branhamella catarrhalis*, an occasional cause of human respiratory infections. This property is absent from most other members of the family *Neisseriaceae*. The butyrate esterase can be detected with fluorogenic 4-MU-butyrate (170, 227). All *Branhamella* strains tested gave a positive fluorescence reaction within 5 min, whereas *Neisseria* spp. remained negative. A test involving a paper strip impregnated with indoxyl butyrate was suggested by Dealler et al. (48) for distinguishing *B. catarrhalis* from other oxidase-positive, gram-negative cocci. Aguirre et al. (2) demonstrated the usefulness of a fluorogenic test for the detection of C₈ esterase directly on colonies grown on MacConkey or salmonella-shigella agars. The test was useful for detection of *Salmonella* colonies.

DNases

Use of synthetic substrates for detection of DNase activity is hampered by the lack of suitable synthetic substrates. The three compounds that are available are more suitable for detection of dinucleotidases or exonucleotidases than endonucleotidases. Tests commonly used for DNase activity based on hydrolysis of natural DNA were previously summarized (16). DNA-containing agar and detection of hydrolysis of DNA by flooding the incubated plate with 1 N HCl was described first (112). Modifications of this, involving tolidine blue (203) and methyl green (210), have advantages because they do not require the addition of reagents after the plates are incubated. Lachica and Deibel (132) used an agar medium containing acridine orange. When cultures were overlaid with an AO DNA-agar mixture, incubated for 1 to 3 h, and observed under UV light, clear halos developed around DNase-positive colonies. Other DNA-binding fluorogenic dyes include 4,6-diamino-2-phenylindole (DAPI) (excitation wavelength, 365 nm; emission wavelength, 390 nm), ethidium bromide (excitation wavelength, 352 nm; emission wavelength, 600 nm), and the Hoechst dye 33258 (excitation wavelength, 352 nm; emission wavelength, 448 nm). Common to these compounds is their weak fluorescence at neutral pH but strong fluorescence when bound to DNA. Such dyes are used to detect DNA fractions on gel electrophoresis and other separative methods; this suggests that fluorescence is present even when the DNA polymer has been catabolized to form oligonucleotides.

Wolf et al. (244) described a rapid testing procedure with a synthetic molecule of 5-bromo-4-chloro-3-indolyl-thymidine-3-phosphate. Thymidine-5-monophosphate-PNP ester ammonium salt was also used by Onishi et al. (162). Hydrolysis of the phosphate ester of 5-bromo-4-chloro-3-indole is

used to indicate DNase activity. Inclusion of this test in a number of multitest kits has been reviewed (16).

Phosphatases

The use of bacterial phosphatases for differentiation of bacterial taxa has been reviewed (16). Both acid and alkaline phosphatases (optimal pHs of 4 to 6 and 8.5 to 9.5, respectively) have been detected. The usefulness of the phosphatase test in distinguishing pathogenic from nonpathogenic strains has been described previously (8, 12, 27, 65, 98, 114, 117, 168, 182, 211, 243, 245).

Synthetic substrates used for the detection of phosphatase activity include derivatives of phenolphthalein (12, 27), phenol (32), α - or β -naphthol, 5-bromo-4-chloro-3-indoxyl (232, 245), PNP (13, 17), and 4-MU (79, 83). Huschtscha et al. (105) described an in situ counting of cells based on fluorogenic assay of alkaline phosphatase. Phosphatase activities of both gram-negative and gram-positive bacteria are detected using 4-MU derivatives in the MicroScan Rapid Identification systems (18).

Peptidases and Proteases

The significance of these enzymes to bacterial characterization was reviewed (16). In earlier studies, derivatives of α - or β -naphthylamides were used and enzymatic activity was detected fluorometrically or by reaction with diazonium dyes (16). Assays with derivatives of p-NA (34, 35, 71, 109) or 7-AMC (71, 79) are more sensitive and easier to perform. Two specific aminopeptidases have been much used, one for specific detection and the other for differentiation between gram-negative and gram-positive bacteria.

Pyroglutamyl aminopeptidase. Pyroglutamyl aminopeptidase (PYRase) (EC 3.4.11.8) activity was found in *Bacillus subtilis* (217). In 1981, Godsey et al. (80) described for the first time the hydrolysis of L-pyrrolidonyl-β-naphthylamide (PYR) as an aid for the identification of group A streptococci and enterococci. Since then, several authors (26, 43, 59, 63, 116, 160, 240) have used PYR for the detection of PYRase activity, mainly for differentiating between these groups of gram-positive cocci. Bosley et al. (26) found that all of the *Enterococcus faecalis* strains, 90% of the *E. faecium* strains, and 96% of the *Streptococcus bovis* biotype I strains examined in their study, were correctly identified by a 4-h method.

Substrates used for detection of PYRase activity include PYR (217), L-pyroglutamyl-p-nitroanilide (71), and L-pyroglutamyl-7-amido-4-methylcoumarin (71).

Rapid methods for detection of PYRase by using impregnated paper strips were described by Wellstood (240) and Kaufhold et al. (116), with the results being available within a few minutes. In another study, the Strep-A-Fluor paper strip test (Bio Spec, Dublin, Calif.), impregnated with a fluorometric substrate, was applied to detection of group A streptococci (235). Monitoring of this bacterial group on agar medium containing PYR has proved to be useful (101). Another method for identification of S. bovis and other related bacteria, developed by Panosian and Edberg (165), combining tests for α-D-GAL, β-D-GLU, and PYRase, enabled the distinction between S. bovis, S. equinus, Enterococcus spp., S. pneumoniae, and the viridans group of streptococci in a culture medium within 30 min. Selective and differential fluorogenic media for the isolation of fecal streptococci were also described by Littel and Hartman (138). In another study (116), two paper strip tests detecting

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PYRase and β -D-GLU activities, were used for rapid identification of *S. pyogenes* and enterococci from human sources within a few minutes.

It is important to remember that tests for one or two enzymes may not be sufficient for unambiguous identification of a specific pathogen when applied directly to clinical or environmental specimens. Facklam (62) suggested that the nonspecificity of tests based on detection of PYRase activity seems to limit the usefulness of such tests in detection of group A streptococci in mixed cultures.

L-Alanine aminopeptidase. L-Alanine aminopeptidase arylamidase has been suggested for differentiating between gram-positive and gram-negative bacteria, being more prevalent in the latter group (34, 35). This distinction reflects a quantitative rather than a definite qualitative difference (16). Kits using p-NA-L-alanine are commercially available (Merck, Darmstadt, Germany; Austin Biological Lab., Austin, Tex.).

Application of the Bactident aminopeptidase test (Merck) has been described by Costin et al. (38) and Otte and Tolle (163). Recently, a comparison was made of usefulness of several tests for distinguishing gram-positive from gramnegative bacteria (144). Traditional Gram staining, lysis by KOH, and use of various chromogenic and fluorogenic substrates for detection of L-alanine arylamidase were examined. Tests were done by using paper strips impregnated with the substrates, or the substrates were incorporated into solid and liquid media. It was found that the fluorogenic substrate L-alanine-7-AMC showed the highest sensitivity.

Other aryl peptidases. Characterization of rhodococci and actinomycetes by using filter papers spotted with substrate solutions was described previously (81). All 105 strains tested showed activity toward derivatives of L-alanine, L-lysine, and L-methionine, L-serine, L-tyrosine, S-benzyl-Lcysteine and L-Ala-Phe-alanine. The rhodococci could be broadly divided into two groups on the basis of activity with derivatives of α-L-glutamic and γ-L-glutamic acids and carbobenzyl-oxy-L-Arg-arginine. Endopeptidase substrates appeared to have better differentiating values than those for aminopeptidases. Bascomb (16) suggested that when a crude extract of a bacterial strain is shown to have arylamidase activities toward a variety of amino acid derivatives, it is not possible to decide, a priori, whether these activities are due to the presence of a single enzyme with broad but varying specificities to the various derivatives or to the presence of a number of enzymes with narrow specificity towards the individual derivatives. The finding (5) that a partially purified preparation of the Tamm-Horsfall glycoprotein showed activity toward a number of p-NA derivatives favors the presence of a single enzyme showing broad and varying specificities.

The activity of proteases such as chymotrypsin (EC 3.4.21.1) and trypsin (EC 3.4.21.4) of bacteria was examined by Gray et al. (86) with 4-MU esters of amino acid derivatives testing the carboxy-terminal specificity of the enzymes. Similar studies were carried out by Kuromizu et al. (130). Detection of trypsin activity in colonies of *Bacteroides gingivalis* by using carbobenzoxy-L-arginine-7-AMC was described by Slots (209).

An improved fluorogenic assay for the rapid detection of *Vibrio parahaemolyticus* was developed (153): its trypsinlike activity was measured with the fluorogenic substrate benzoyl-L-arginine-7-AMC.

Correlation between peptidase/protease activities on synthetic substrates and natural substances has not been conclusive (16). Recently, Homer et al. (100), studying the use

of enzymatic tests for differentiation between Streptococcus species, showed a good correlation between activity toward synthetic 7-AMC endopeptidase substrates and hydrolysis of natural substances. Streptococcus oralis and Streptococcus sanguis showed the highest, most varied proteolytic activity.

Coagulase

The ability to produce coagulase is the most widely used criterion for identification of Staphylococcus aureus. It can be performed as either a tube or slide coagulation test. The method is based on the ability of both free and bound coagulase to clot plasma. Staphylocoagulase reacts with prothrombin to form the complex staphylothrombin, which converts fibrinogen into insoluble fibrin, leading to clotting of blood. Engels et al. (60) showed that, in the presence of prothrombin, hydrolysis of Chromozym TH (N-tosyl-glycyl-1-prolyl-L-arginyl-p-nitroanilide hydrochloride) can be used as a measure for coagulase activity by the detection of the released p-NA. For direct assay of S. aureus, Bulanda et al. (29) evaluated the chromogenic substrate D-Phe-Pro-Arg-βnaphthylamide HCl for the detection of staphylocoagulase. Release of \(\beta\)-naphthylamine was demonstrated by addition of a diazo dye.

USE OF FLUOROGENIC DYES

The increase in the fluorescence of dyes when adsorbed to macromolecules has been used for detection, enumeration, and differentiation of bacteria. Fluorogenic dyes have been incorporated in growth media to enable the differentiation between gram-negative and gram-positive colonies. Such dyes have also been used in combination with magnification and counting techniques for extension of knowledge to the level of individual cells. The two important fields in which fluorogenic dyes are used are FCM and DEFT.

In DEFT, described by Pettipher and coworkers (141, 173–175), samples are first treated to disperse cell and other aggregates and to increase efficiency of filtration. The microbial content of the sample is concentrated by filtration onto polycarbonate membranes and application of vacuum. The concentrated sample is then stained with fluorogenic nucleic acid dyes. Epifluorescence microscopy combined with various degrees of image analysis is used to enumerate the number of fluorescent particles. A combination of epifluorescence microscopy and staining with a tetrazolium salt was used by Zimmerman et al. (247) for simultaneous determination of the total number of bacteria and the number showing respiratory activity.

In FCM, particles are measured while passing through a highly focused light beam. The use of fluorogenic reagents enables measurement of specific cell constituents such as proteins, DNA, RNA, surface antigens, and specific nucleotide sequences. The ability of FCM to perform simultaneous measurements of a number of cell constituents, as well as to determine particle size, makes the system an exciting and interesting microbiological tool for characterization of cell populations (70, 152, 205).

Unfortunately, the small size of bacterial cells requires expensive equipment; thus, application of the technique to bacterial studies has not progressed as much as in other biological fields (70). Use of fluorogenic substrates for detection of enzymatic activity of individual bacterial cells has been very limited (40, 70).

Protein-Specific Dyes

A medium containing the fluorogenic compound ANS for simultaneously enumerating gram-negative and gram-positive bacteria and for differentiating between various species was reported by Ramsey et al. (185) as well as by Kwee et al. (131). The mechanism of staining is thought to be that of adsorption of ANS to bacterial cell wall proteins in growing cultures (167). Aithal et al. (3) studied the effects of temperature and respiratory inhibitors on the affinity of electron transport particles of Mycobacterium phlei to ANS. The effect of the temperature of cells on the fluorescence intensity of two gram-negative and two gram-positive cultures during measurement was described by Mäntsälä (146). A decrease in fluorescence intensity was observed when the temperature was elevated from 4 to 40°C. A strong increase in intensity was observed with cells of Pseudomonas fluorescens and E. coli, but only a slight increase was observed with cells of Streptococcus thermophilus or B. subtilis. ANS at high concentration is capable of inhibiting the growth of most gram-positive but not gram-negative microorganisms. According to Ramsey et al. (185), the light emitted by colonies of the gram-negative strains is much more intense than that emitted by gram-positive ones. Furthermore, the intensity of light emission of various bacteria differed with the wavelengths.

Neisseria meningitidis can be differentiated from Neisseria gonorrhoeae by using ANS. N. gonorrhoeae is extremely sensitive to ANS; growth is inhibited in ANS-containing media (218). Characteristics of fluorescent reagents for the visualization of eucaryotic cell constituents were described by Waggoner et al. (233).

Nucleotide-Specific Dyes

Dyes that are weakly fluorescent at neutral pH but strongly fluorescent when adsorbed onto DNA, such as ethidium bromide, have been used for the detection of aquatic microflora (reviewed in reference 16). Direct counting of aquatic bacteria concentrated on membrane filters stained with fluorochromes by using fluorescence (133) and epifluorescence microscopy (95, 246) has been described. A method for enumeration of bacteria in milk, based on adsorption of AO, has been developed (174, 175). Bacterial cells stained with AO may show orange-red or green fluorescence. Orange-red fluorescence has been attributed to viable cells, abundance of RNA, or heat-denatured DNA. Green fluorescence has been attributed to nonviable cells and DNA (104). The importance of acid pH in differentiating between human cells (green) and bacteria (orange) has been described previously (129). Cream coloration indicates the presence of both red and green fluorescence (95). The special application of AO staining combined with microscopy, for direct determination of the viable count, has been described previously (125, 128, 194, 195, 197). DEFT-type methods have been developed for foods (196), in breweries (47), and in wineries (37). Arndt-Jovin and Jovin (10) described and discussed the factors affecting fluorescent staining of DNA molecules of living eucaryotic cells. FCM techniques involving nucleic acid-specific dyes such as AO, ethidium bromide (178, 216), DAPI, Hoechst 33258 (139), mithramycin (216), and propidium iodide (52, 200, 225, 231) have been used for rapid counting of bacteria in pure cultures (178, 216) and in clinical (225), environmental (225), food (52), and industrial specimens. DAPI and Hoechst 33258 dyes bind preferentially to adenine-thiamine (A · T) base pairs, whereas chromomycin A3 and mithramycin are specific to guanine-cytosine (G · C) base pairs (10, 139, 202, 205, 226). A combination of such dyes has been used to distinguish between populations of bacteria markedly different in G+C content such as $E.\ coli,\ P.\ aeruginosa,\ and\ S.\ aureus$ (205). However, populations of $E.\ coli$ and $Klebsiella\ pneumoniae$ could not be separated (202). The method has also been shown to provide a means of rapid determination of the G+C content of DNA in pure cultures (202).

FCM techniques in conjunction with protein-specific dyes such as fluorescein isothiocyanate (107), fluorescence-labeled antibodies (52, 107, 231), and nucleotide sequences (7), have been used for detection and enumeration of certain bacterial taxa. These include *E. coli* (216), *L. pneumoniae* (107), other *Legionella* spp. (225), *Nitrosomonas* spp. (231) and *Streptococcus pyogenes* (200). As few as 5×10^3 and 5×10^4 CFU of *S. pyogenes* per ml were enumerated in buffer and saliva, respectively. Tyndall et al. (225), using fluorescein iodothiocyanate-labeled specific antibodies and propidium iodide, could detect *Legionella* spp. in samples from water-cooling towers.

APPLICATION OF ENZYME TESTS TO SPECIFIC TAXA

The use of enzyme test kits and application of batteries of enzyme tests for characterization of individual genera have been summarized by Bascomb (16).

Differentiation of Members of the Family Enterobacteriaceae

Further enzymatic tests were described for the examination of different genera of the family Enterobacteriaceae (28, 73–75). Rambach (184) described a new agar medium for the differentiation of Salmonella spp. The medium contains propylene glycol and neutral red as the pH indicator for detection of Salmonella colonies, which appear bright red. The medium also contains X-Gal for the detection of $E.\ coli$ and other β -GAL-positive colonies, which appear blue. Colonies of $Proteus\ mirabilis\ and\ Morganella\ morganii\ appear\ colorless.$

The evaluation of a rapid commercially available method, the MUCAP test (Biolife Italiana S.R.L., Milan, Italy) intended for the screening of Salmonella spp., has been reported (2, 181). This test is based on the detection of C_8 esterase enzyme by using a fluorogenic 4-MU-containing substrate.

Chromogenic substrates for peptidases were used for the detection of *Shigella* serovars and biovars. Chromozym PL (77) and Chromozym U (147) allowed the differentiation of *Shigella* strains. *Proteus* and *Providencia* strains in clinical specimens were detected by using *p*-nitro-DL-phenylalanine (PNPA). Bascomb and Spencer (17) described the use of PNPA for detection of the tribe *Proteae*. A 25-min test, using continuous-flow equipment capable of performing 20 tests per h, showed that only the members of the tribe *Proteae* gave positive results. A 2-h test (16) and macro- and microtests (76) have been described.

Identification of Legionella Species

Berdal et al. (21) demonstrated that synthetic chromogenic peptide substrates can be used to detect extracellular proteolytic enzymes from Legionella spp. L. pneumophila was differentiated from other Legionella species on the basis of the qualitative and quantitative differences in hydrolysis of

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four oligopeptide derivatives of p-NA. Another study presented by Berdal et al. (20) showed that proline-specific endopeptidase activity was present in Legionella and Flavobacterium cultures. These reactions can be shown by using peptide derivatives of p-NA. However, a 100-fold concentration step was needed to achieve a detectable amount of protease activity. Diazotization of p-NA improved the sensitivity of the assay (22). The value of several biochemical tests for the identification of L. pneumophila was compared by Vesey et al. (229), who showed that 23 of 42 attributes were useful. Alkaline phosphatases, catalase, peroxidase, lipase, and proteolytic activities, as detected by a number of natural substrates, were of particular value. Similar results were obtained by Müller (158), using a commercially available API ZYM system.

Identification of Neisseria Species

A number of enzyme tests have been found useful (45, 214, 246). Behal and Cox (19) studied the peptidase activity of Neisseria catarrhalis and reported that an intracellular enzyme showed higher affinity toward β-naphthylamide-Ala than toward Ala-Ala peptide. D'Amato et al. (45) selected 10 enzyme tests. The tests most frequently used are y-Lglutamyl arylpeptidase and L-hydroxyproline arylpeptidase (61, 237). The first test is specific for identification of N. meningitidis (45, 97, 190); the second is specific for N. gonorrhoeae (45, 97, 102). The β -GAL test is specific to N. lactamica. The production of hydroxyprolylaminopeptidase by N. cinerea, but not by B. catarrhalis, is of possible differential value (97). Ison et al. (109) studied the specific activities of y-L-glutamyl- and L-hydroxylprolyl-arylamidases of N. gonorrhoeae and two populations of N. meningitidis, one consisting of strains isolated from systemic infection and the other consisting of strains isolated from the throats of male patients attending a sexually transmitted disease clinic. The pharyngeal meningococci showed a lower specific activity of γ-L-glutamyl-arylamidase and higher specific activity of L-hydroxyprolyl-arylamidase, suggesting the occurrence of genetic interchange between the two species.

Enzyme Tests in Mycobacterial Taxonomy

The use of umbelliferone derivatives in the study of mycobacteria was described by Grange (83), Grange and Clark (84), and Slosárek (208). They showed that 4-MU substrates provide a very sensitive method for detecting mycobacterial group-specific hydrolases, such as glucosidases, hexosaminidases, acylesterases, lipases, phosphatases, and arylsulfatases. It was further shown that substances containing 4-MU were particularly suitable for examining the glycosidase activity of slowly growing mycobacteria (85). Mycobacterium nonchromogenicum, M. szulgai, and M. marinum could be differentiated by their α-L-fucosidase activity (85).

Enzyme Tests in Fungal Taxonomy

Slifkin and Cumbie (207) described the use of Congo red as a fluorochrome for the rapid detection of fungi. The stain may be prepared with potassium permanganate as a counterstain or with formalin or glutaraldehyde (207). Bobey and Ederer (25) demonstrated that different enzymes of yeasts can be monitored using 4-MU substrates, following the same principle as described for bacteria. In particular, detection of

phosphatase, β-D-GLU, and pyrophosphate-diesterase can contribute to differentiation.

Identification of Candida Species

Candida albicans is the most frequently isolated yeast pathogen. It was differentiated from Candida parapsilosis by using p-nitrophenyl-N-acetyl-β-D-galactosaminide for detection of N-acetyl-β-D-galactosaminidase (EC 3.2.1.30) (119). Perry and Miller (171) and Dalton et al. (42) found that 4-MU-N-acetyl-β-D-galactosaminide could be used for rapid detection of C. albicans; 99.7% of the C. albicans and only 7% of Candida tropicalis strains tested showed a positive reaction. Smitka and Jackson (212) developed a fluorogenic test for differentiating the C. parapsilosis group from other Candida species. They used 4-MU-\(\beta\)-D-GLU to assay the β-glucosidase activity spectrophotometrically within 30 min. All C. parapsilosis strains were positive. In contrast, preliminary results obtained with a substrate buffer system of low pH had supported the findings of Bobey and Ederer (25) that, under certain conditions, \(\beta\)-glucosidase activity was not significant in C. parapsilosis. Hydrolysis of glycosides can be affected by enzyme specificity and by reaction conditions. The results of Polachek et al. (180) illustrate that a reliable differentiation of C. albicans cannot be performed only on the basis of β -D-glucosidase activity.

RAPIDITY OF TEST PERFORMANCE

Many tests for specific detection of pathogens or indicator organisms are described as rapid. When considering the duration of a test, it is important to take into consideration the size of inoculum required and the effect of the recent history of the culture on test results. Thus, a test that requires growth from a number of colonies or a turbidity equivalent to a McFarland standard of ≥1 often requires a pure culture; this adds an overnight incubation period to the duration of the test. Therefore, the so-called 15-min test in reality may require 24 h. The recent history of the culture is also important. Media used for primary isolation may inhibit the synthesis of enzymes of interest. Tests for preformed enzymes may give different results depending on the medium on which the culture was grown prior to the test. For example. Iritani and Inzana (108) describe a rapid (30- to 60-min) tube assay for detection of E. coli based on detection of the GUD and β-GAL enzymes and indole. However, the test could be performed only with bacteria taken from blood agar. Most isolates of E. coli grown on MacConkey agar were not identified correctly within 60 min by the tube assay. Those enzyme tests that rely on subjective observation for determination of results may be either growth-dependent tests or spot tests. In the first type, synthetic substrates are incorporated into liquid or solid growth medium; determination of results takes place after growth has occurred. In the second type, bacterial suspensions are added to substrates dried on filter papers or cotton swabs; results are observed after 1 to 20 min. In these spot tests (so-called rapid tests), bacteria from one or more colonies are required. If more than one test is required to characterize the isolate unequivocally, a pure culture may be needed. On the other hand, tests based on measurement of enzymatic activity by photometers or fluorometers can detect activity with a smaller inoculum.

CONCLUSIONS AND FUTURE PERSPECTIVES

Rapid detection and identification of microorganisms is of great importance in a diverse array of applied and research fields. By incorporation of synthetic enzyme substrates into primary isolation media, enumeration and detection can be performed directly on the isolation plate. Alternatively, enzyme tests can be performed on suspensions of nonproliferating bacterial cells. In the latter case, the method of detection of enzymatic activity will determine the number of cells required. Visual detection, as is practiced with many spot tests, requires more cells than spectrophotometric methods. Visual detection in mixtures of bacteria and liquid substrates requires longer incubations than spectrophotometric assays.

In general, fluorogenic and chromogenic substrates have proved to be a powerful tool, utilizing specific enzymatic activities of certain microorganisms, either in addition to or instead of traditional methods. Many of them are offering enhanced accuracy and performance to the microbiologist. Enzyme tests have been incorporated in a number of commercially available test kits. For instance, testing systems such as the MicroScan Rapid Identification panels, based on fluorogenic tests, provide identification of both gram-positive and gram-negative bacteria within 2 h (118); the Sensititre Autoidentification system provides identification of gram-negative bacteria within 5 or 18 h (14, 221).

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